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Antioxidant Assessment of *Pleurotus sajor-caju* Extracts Obtained through Hot Extraction Process

Gupta K.K[1], Maurya S[1], Agarwal S[1], Kushwaha A[1], Kumar R[2], Pandey A.K[2], Singh A.K[1], Verma V[1] and Singh M.P[1]*

[1]*Centre of Biotechnology, University of Allahabad, Allahabad, India*
[2]*Department of Biochemistry, University of Allahabad, Allahabad, India*

Abstract

Current work reports antioxidant activities of extracts derived from *P. sajor-caju* using several *in vitro* models. Dried samples were sequentially extracted in hexane, ethyl acetate, methanol and water using hot extraction method. Extraction with hexane and ethyl acetate resulted in negligible recovery. Methanol and water extracts tested positive for phenolics, flavonoids, terpenoids, anthraquinones and saponins. Total phenol and flavonoid contents were found in the range of 19.78-23.59 µg propyl gallate equivalent/mg and 23.27-26.44 µg quercetin equivalent/mg in aqueous and methanolic extracts, respectively. Comparatively lower activities were observed for both the extracts in DPPH radical scavenging and reducing power assays. Appreciable metal ion chelating activity was demonstrated by aqueous (57% to 86%) and methanolic (36% to 71%) extracts in the concentration range 100-400 µg/ml, showing dose dependent response. Total antioxidant capacity for both the extracts was found in the range 83-96 µg PGE/ml at test concentration. The study showed *P. sajor-caju* aqueous and methanolic extracts have antioxidant potential.

Keywords: *Pleurotus sajor-caju*; Extracts; Antioxidant; Radical scavenging; Reducing power; Metal ion chelation

Abbreviations: *P. sajor-caju*: *Pleurotus sajor-caju*; DPPH: 2,2-Diphenyl-1-Picrylhydrazyl; MeOH: Methanol; AQ: Water

Introduction

P. sajor-caju is an edible mushroom which is commonly known as oyster mushroom. This mushroom possesses all the three properties of food i.e., nutrition, taste and physiological function [1]. *Pleurotus* confers advantages over other mushrooms for its capability to grow on non-fermented lingocellulosic wastes and produce in turn fruit bodies with higher nutritious content [2,3]. Mushrooms are attracting attention of people all around the globe because of their nutritional and medicinal attributes.

As a low calorie, high protein diet with almost no starch and sugars, mushrooms are the delight of the diabetic. Due to high K: Na ratio, few calories and low fat (rich in linoleic acid and lacking cholesterol), mushrooms are the choice of the dietician for those with obesity, hypertension, atherosclerosis and heart disease [4].

Free radicals are associated with many degenerative diseases including cancer, cardio-vascular diseases, cataract, and immune system decline and brain dysfunction. Potentially harmful reactive oxygen species (ROS) are produced as the consequence of normal aerobic metabolism. Under normal conditions about 2% to 5% of O_2 consumed by mitochondria is converted to ROS during metabolic process [5]. Antioxidants neutralize the adverse effects of free radicals/ROS. They usually inactivate ROS *in vivo* by scavenging them or preventing their generation and thereby minimizing the oxidative damage. Deficient antioxidant defenses or overproduction of ROS may lead to oxidative stress, which might be associated with a variety of degenerative disorders [6]. Antioxidants are known to inhibit LDL oxidation in cell-dependent and cell-free systems which may provide protection from oxidative stress [7]. Synthetic antioxidants used in the food industries have been implicated in liver damage and carcinogenesis. Hence supplementation of the diet with plant derived natural antioxidants might serve as preventive medicine [8].

Several groups have reported biological activities of *P. sajor-caju* extracts obtained through cold extraction method using limited number of solvents [9]. Present communication reports the biochemical composition and antioxidant activities such as radical scavenging, metal ion chelating and reducing power activities of sequential extracts derived from *P. sajor-caju* using hot extraction process.

Materials and Methods

Culture and its maintenance

The pure culture of *Pleurotus sajor-caju* was procured from Directorate of Mushroom Research, Solan (HP), India. This was maintained on Malt Extract Agar (MEA) medium at 25 ± 2°C and pH 6-6.5 sub cultured at regular interval of three weeks.

Spawn preparation

Spawn is referred to as the vegetative mycelium of the fungus, which is grown on cereal grains. Wheat grain spawn was prepared by the following method of Singh et al. [10] which is well established in author's laboratory and has been detailed in the paper [3].

Preparation of substrate

The paddy straw which was used as substrate to cultivate the mushroom was completely dipped in water. The substrate was allowed to stay in water for overnight. After that excessive water was drained out. After draining, the paddy straw was again completely dipped in hot water (temperature 70-80˚C) for an hour. Then excess water was drained out.

Spawning

Spawning is the process of mixing spawn in the sterilized substrates.

***Corresponding author:** Singh M.P, Centre of Biotechnology, University of Allahabad, Allahabad-211002, India, Tel: 919415667798; E-mail: mpsingh.16@gmail.com

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5% wet weight basis spawn grain was mixed with the substrate and filled into polythene bags. The mouth of the bag was tied with rubber band and 12 holes of about 1cm diameter were made; two at each corner of the base, four each on the broader area and one each on the narrow, rectangular side to drain out extra water and for proper aeration [11].

Mushroom sample collection

Fruiting bodies were collected and air dried. Dried materials were crushed and ground into fine powder with mortar and pestle and it was kept under refrigeration at 4°C for further analysis.

Preparation of extracts

Powdered sample was sequentially extracted with hexane (HX), ethyl acetate (EA), Methanol (MeOH) and water (AQ) in Soxhlet apparatus for 8 h [12]. Solvent was removed completely under reduced pressure and dried extracts were preserved at −20°C. The dried residues were reconstituted in DMSO for determination of biochemical activities.

Biochemical screening

Qualitative biochemical analysis was carried out for identification of flavonoids, terpenoids, saponins, anthraquinone, tannin and phlobatannins in *P. sajor-caju* extracts using standard procedures [13].

Determination total flavonoid content

Aluminum chloride colorimetric method [14] with minor modification was used for determination of flavonoid content in extract fractions. Small amount (0.2 ml) of extract in pure DMSO (2 mg/ml) was separately mixed with 1.8 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. Tubes were incubated at room temperature for 30 min and then absorbance of the reaction mixture was measured at 415 nm. Calibration curve was prepared using quercetin as standard compound. The amount of flavonoids in the test samples was expressed as µg quercetin equivalent/mg sample (µg QE/mg). Experiments were performed in triplicate and the results were expressed as mean ± SD.

Determination of total phenolics

Total phenolic content in extract fractions was determined according to the protocol [15] with some modifications [16]. Modification included dissolution of extracts in DMSO instead of water. 0.2 ml of sample (2 mg/ml in DMSO) was diluted to 3 ml with water. Small amount (0.5 ml) of two-fold-diluted FCR was added and the contents were mixed. After 3 min, 2 ml of 20% sodium carbonate solution was added and the tubes were placed in boiling water bath for one min followed by cooling. The absorbance was measured at 650 nm. The concentration of phenols in the extract samples was expressed as µg propyl gallate equivalents/mg sample (µg PGE/mg). The estimation was performed in triplicate, and the results were expressed as mean ± SD.

DPPH radical scavenging activity

The free radical scavenging activity of the extract fractions was measured *in vitro* by DPPH assay [15] with minor modification [16]. DMSO was used as solvent for dissolving extracts instead of methanol. Three milliliters of 0.1 mM DPPH solution prepared in methanol was added to 1 ml of the test extracts (40-100 µg/ml) dissolved in DMSO. The content was mixed and allowed to stand at room temperature for 30 min in dark. The reduction of DPPH free radical was measured by recording the absorbance at 517 nm. The percentage scavenging activities (% Inhibition) at different concentrations of the extracts were calculated using the following formula.

$$(\%)\ I = ((Ac - As)/Ac) \times 100$$

Where I is inhibition, *A*c and *A*s are the absorbance values of the control and the sample respectively. Three replicates were made for each sample and results were expressed as mean ± SD.

Reducing power assay

The reducing power of test extracts was determined by the protocol [17]. One ml aliquots of extracts (200-1000 µg/ml) prepared in DMSO was taken in test tubes. To each test tube 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml of 1% potassium hexacyanoferrate ($K_3Fe(CN)_6$) were added and contents were mixed. Tubes were incubated at 50°C in a water bath for 20 min. The reaction was stopped by adding 2.5 ml of 10% TCA and then centrifuged at 4000 g for 10 min. One ml of the supernatant was mixed with 1 ml of distilled water and 0.5 ml of $FeCl_3$ solution (0.1%, w/v) and kept at 25°C for 2 min. The absorbance was measured at 700 nm. Higher absorbance indicated higher reducing capability of the sample. All the tests were run in triplicate and results were reported as mean ± SD.

Metal ion chelating activity

The chelation of ferrous ions by the *P. sajor caju* extracts was estimated by the method of Dinis et al. [18] as modified by us. Modification included dissolution of extracts in DMSO instead of methanol. Briefly, the extract samples (200 µl) of different concentrations were added to a solution of 2 mM/L ferrous sulphate (0.05 ml). The reaction was initiated by the addition of 5 mM ferrozine (0.2 ml) and the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. Metal ion chelating ability (percentage inhibition of ferrozine-Fe^{2+} complex formation) was calculated using the formula.

$$\text{Metal ion chelating ability } (\%) = ((Ac - As)/Ac) \times 100$$

Where *A*c is the absorbance of control and *A*s is absorbance in the presence of the sample. The results were expressed as mean ± SD of three replicates.

Phosphomolybdate assay

The total antioxidant capacity of the extracts was determined by Phosphomolybdate method using propyl gallate as standard [19] with some modification [16]. To 50 μL extract solution (containing 100 μg dried extract) prepared in DMSO, 0.25 ml methanol was added followed by the addition of 3 ml of reagent (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min leading to development of green colour. After the samples got cooled to room temperature, the absorbance was measured at 695 nm. The total antioxidant (AO) capacity was expressed as μg propyl gallate equivalents per milligram of sample (μg PGE/mg of sample). The results were expressed as mean ± SD ($n = 3$).

Statistical Analysis

All experiments were carried out in triplicate and data were represented as mean ± standard deviation (SD). Graphs were prepared using Graph Pad Prism software.

Results

Biochemical analysis of extracts

Recovery of extracts was almost negligible in hexane and ethyl acetate fractions. Hence all the tests were performed on methanol and

Biochemicals	Methanol Extract	Aqueous Extract
Tannins	-	-
Flavonoids	+	+
Terpenoids	+	-
Anthraquinones	+	+
Phlobatannins	-	-
Saponins	+	+

Table 1: Biochemical screening of *P. sajor-caju* extracts.

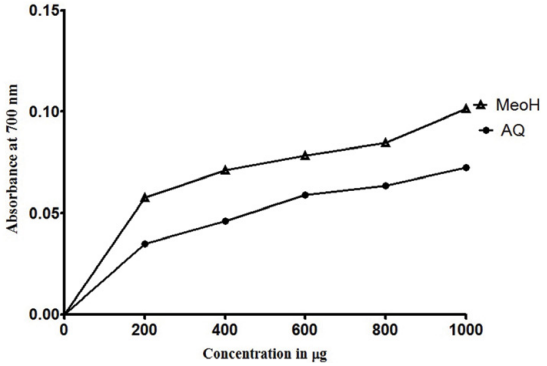

Figure 1: Reducing power assay of *P. sajor-caju* extracts. (Abbreviations: MeOH: Methanolic Extract; AQ: Aqueous Extract.

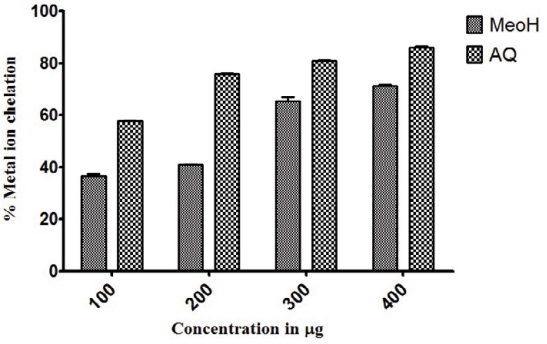

Figure 2: Metal ion chelating activity of *P. sajor-caju* extracts.

aqueous extracts only. Biochemical screening of *P. sajor-caju* extracts exhibited presence of flavonoids, terpenoids, reducing sugars, saponins, anthraquinone in MeOH and AQ fractions (Table 1). Tannin and phlobatannins were absent in extracts.

Total phenolic and flavonoid contents

Total phenolic contents in AQ and MeOH extracts were 19.78 ± 0.59 µg PGE/mg and 23.59 ± 0.99 µg PGE/mg of samples, respectively. Total flavonoids in both the extracts ranged between 23.27-26.44 µg QE/mg of sample.

DPPH assay

P. sajor-caju extracts (AQ and MeOH) showed 2-6% DPPH free radical scavenging potential at test concentrations. Antioxidants react with DPPH free radical and change the color of reaction mixture from violet to yellow. Results demonstrated that both extracts have low capability to donate hydrogen atom.

Reducing Power assay

Reducing power ability of test compounds exhibited the similar pattern as observed in radical scavenging assay (Figure 1). *P. sajor-caju* MeOH extract showed moderate reducing ability (Absorbance 0.06-0.11). AQ extract also showed lower activity (Absorbance 0.03-0.07) in the concentration range 200-1000 µg/ml.

Metal ion chelation

Test extracts displayed concentration dependent metal chelation potential (Figure 2). Aqueous extracts accounted for appreciable metal ion chelating ability (57-86%). Methanolic extracts also showed noticeable chelation activity (36-71%) at test concentrations. Transition metals are responsible for increasing ROS generation in the system. Hence chelation of iron by *P. sajor-caju* extracts shows their ability to diminish ROS production.

Phosphomolybdate assay

The AQ and MeOH extracts derived from *P. sajor-caju* exhibited noticeable AO capacity. The AO capacity of AQ and MeOH extracts was 83.68 ± 0.60 μg PGE/mg and 95.89 ± 0.58 μg PGE/mg of extract, respectively. Phosphomolybdate assay is used for assessment of total antioxidant capacity of samples.

Discussion

Pleurotus species are promising as medicinal mushrooms and exhibit hematological, antiviral, antitumor, antibacterial, hypocholesterolic, and immunomodulatory activities as well as antioxidant properties. Approximately 40 species of the oyster mushrooms (genus *Pleurotus*) have been reported in the literature. Scientific studies on macrofungi (mushrooms and entomopathogenic fungi) have reported that extracts derived from fruiting bodies or mycelia possess important medicinal properties [20-22]. In the present work, biochemical analyses of fruiting bodies of *P. sajor-caju* has shown presence of number of bioactive compounds such as flavonoids, terpenoids, reducing sugars, saponins, anthraquinone (Table 1). Considerable amount of phenolics and flavonoids have been found in AQ and MeOH extracts. These compounds are known for their medicinal attributes including antioxidant properties [23].

Current work also describes the antioxidant properties of methanolic and aqueous extract of *P. sajor-caju*. Antioxidants counteract the adverse effects of free radicals in the body by scavenging or reducing their formation [5]. DPPH assay is one of the most commonly used methods for screening antioxidant activity of plant extracts [24]. The hot extracts accounted for lower DPPH radical scavenging activity in the experiments which contradict the reports showing higher radical scavenging activity in cold extracts [25]. Lower radical scavenging activity in our study might be due to a chemical alteration in the active compounds present in this mushroom caused by using a higher temperature during hot extraction process. In reducing power assay MeOH and AQ fractions showed moderate activity even at higher concentrations (Figure 1). Dose dependent response in activity pattern was also observed [17]. Lower proton donating ability marks lower scavenging action. Present study exhibited direct correlation between lower radical scavenging actions of hot extracts with lower reducing power.

The transition metal ion, Fe^{2+} possess the ability to move single electrons, by which it can allow the formation and propagation of many radical reactions, even starting with relatively non-reactive radicals. The main strategy to avoid ROS generation that is associated with redox active metal catalysis involves chelating of the metal ions [26]. Ferrozine can quantitatively form complexes with Fe^{2+}. In the

presence of *P. sajor-caju* extracts formation of red coloured complexes decreased substantially (Figure 2). Therefore, measurement of the rate of color reduction helps to estimate the chelating activity of the coexisting chelator present in the samples. Our results have shown that the absorbance of coloured complex decreased linearly which indicated that the formation of Fe^{2+}-ferrozine complex was incomplete in the presence of *P. sajor-caju* extracts suggesting chelation of iron by biochemicals present in these extracts.

The phosphomolybdenum method is based on the reduction of molybdenum (VI) by the antioxidants and the formation of a green molybdenum (V) complex, which shows maximum absorbance at 695 nm [27]. The difference in AO capacity of different extracts may be attributed to differences in their chemical compositions. Total AO capacity shown by AQ and MeOH extracts of *P. sajor-caju* extracts was in the range 83.68-95.89 μg PGE/mg of extract signifying noticeable antioxidant activity. The antioxidant activity shown by our extracts could be attributed to the presence of flavonoids, polyphenols, and tannins [9]. Medicinal activity coupled with nutritional components present in *P. sajor-caju* could be one reason for its wide use in society.

Conclusion

The bioconstituents present in *P. sajor-caju* extracts have shown promising antioxidant activity viz., metal ion chelating activity and total antioxidant capacity in the present work. Hence results validate the use of edible mushroom *P. sajor-caju* as medicinal food.

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